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Kratzer, A ; Giral, H ; Landmesser, U

**Abstract:** Alteration of endothelial cell functions, including reduced endothelial nitric oxide (NO) availability, increased endothelial cell apoptosis, adhesion molecule/chemokine expression and pro-thrombotic activation are thought to contribute to the pathophysiology of atherosclerosis and coronary-artery-disease (CAD) with its clinical complications, such as acute coronary syndromes. High-density lipoproteins (HDL) from healthy subjects or reconstituted HDL have been observed to exert potential direct anti-atherogenic effects by modulating these endothelial cell functions. Importantly, endothelial effects of HDL have now been reported to be highly heterogeneous, and are modulated as part of immune responses. More recently, this has also been observed for HDL of patients with CAD, where HDL becomes potentially pro-inflammatory and endothelial-protective properties are markedly altered. Several mechanisms may lead to these altered endothelial effects of HDL in patients with CAD, including oxidative modification of HDL-associated lipids and proteins, such as apoA-I and paraoxonase-1, and alterations of HDL-proteome. These findings have to be considered with respect to interpretation of recent clinical studies failing to demonstrate reduced cardiovascular events by HDL-cholesterol raising strategies in patients with CAD. Both clinical and genetic studies suggest that HDL-cholesterol levels alone are not a sufficient therapeutic target in patients with CAD. The focus of this review is to summarize the role of HDL onto endothelial homeostasis and to describe recently characterized molecular pathways involved. We highlight how structural and functional modifications of HDL particles in patients with CAD may perturb the physiological homeostasis and lead to a loss of endothelial-protective properties of HDL in patients with CAD

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# High-density lipoproteins as modulators of endothelial cell functions: alterations in patients with coronary artery disease

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Alteration of endothelial cell functions, including reduced endothelial nitric oxide (NO) availability, increased endothelial cell apoptosis, adhesion molecule/chemokine expression and pro-thrombotic activation are thought to contribute to the pathophysiology of atherosclerosis and coronary-artery-disease (CAD) with its clinical complications, such as acute coronary syndromes. High-density lipoproteins (HDL) from healthy subjects or reconstituted HDL have been observed to exert potential direct anti-atherogenic effects by modulating these endothelial cell functions. Importantly, endothelial effects of HDL have now been reported to be highly heterogeneous, and are modulated as part of immune responses. More recently, this has also been observed for HDL of patients with CAD, where HDL becomes potentially pro-inflammatory and endothelial-protective properties are markedly altered. Several mechanisms may lead to these altered endothelial effects of HDL in patients with CAD, including oxidative modification of HDL-associated lipids and proteins, such as apoA-I and paraoxonase-1, and alterations of HDL-proteome. These findings have to be considered with respect to interpretation of recent clinical studies failing to demonstrate reduced cardiovascular events by HDL-cholesterol raising strategies in patients with CAD. Both clinical and genetic studies suggest that HDL-cholesterol levels alone are not a sufficient therapeutic target in patients with CAD. The focus of this review is to summarize the role of HDL onto endothelial homeostasis and to describe recently characterized molecular pathways involved. We highlight how structural and functional modifications of HDL particles in patients with CAD may perturb the physiological homeostasis and lead to a loss of endothelial-protective properties of HDL in patients with CAD.

**Keywords** HDL • Endothelial cells • Atherosclerosis • Coronary artery disease

This article is part of the Spotlight Issue on HDL biology: new insights in metabolism, function, and translation.

## 1. Endothelial cell function and atherosclerosis

The endothelium, the inner cellular lining of blood vessels and lymphatics, plays a key role for vascular homeostasis beyond its barrier function, e.g. by controlling vascular tone, immune cell recruitment for innate and adaptive immune responses, haemostasis and angiogenesis.<sup>1–4</sup> Alterations in endothelial cell functions, including a reduced endothelial cell nitric oxide (NO) availability, endothelial cell pro-inflammatory activation, increased endothelial cell apoptosis, and pro-thrombotic activation have been suggested to contribute to vascular pathology, in particular to the development of atherosclerosis and coronary artery disease with its clinical sequelae.<sup>4,5</sup>

Of note, endothelial cells have a marked phenotypic heterogeneity in structure and function across the vascular tree<sup>6–8</sup> and vary between

different organs and blood vessel types, in part due to different tissue environments and epigenetics.<sup>2,6–11</sup> A better understanding of underlying mechanisms may aid in the development of vascular bed-specific therapies.<sup>2,6–11</sup>

Common cardiovascular risk factors, such as smoking, hypertension, hypercholesterolaemia, type-2 diabetes, and ageing are associated with altered arterial endothelial cell functions, including an impaired endothelium-dependent vasodilation.<sup>1,3,4,12–15</sup> Clinical studies have observed that altered endothelium-dependent vasodilation, in part due to a reduced endothelial NO availability, is associated with an increased risk of adverse cardiovascular events.<sup>5,16,17</sup>

In addition to the well-known pro-atherogenic role of low-density lipoprotein (LDL) with its potential adverse effects on endothelial cell functions, there is now an increasing interest towards a better understanding of the vascular effects of high-density lipoprotein (HDL), that

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has been observed to exert direct effects on endothelial cell function, that have been suggested to be potentially anti-atherogenic, when HDL was isolated from healthy subjects,<sup>18–21</sup> but are highly heterogeneous when HDL from patients with coronary disease is examined.<sup>22–24</sup>

## 2. HDL and its relation to coronary disease

HDL, a small lipoprotein (7–17 nm diameter), is constituted of an outer layer of phospholipids (PL) and free cholesterol (FC) stabilized by apolipoproteins (2–7 per particle) and a lipid core containing cholesterol esters (CE) and triglycerides (TG).<sup>25–27</sup> Relative proportions of HDL components are modified along its biogenesis by the action of several enzymes and interactions with specific receptors.<sup>28</sup> ApoA-I, the main HDL protein, is mainly synthesized by hepatocytes and in the intestine and associates PL and FC to form lipid-poor complexes, most often depicted as discoidal particles.<sup>28</sup> The enzyme lecithin cholesterol acyl-transferase (LCAT) enriches the lipid core pool with CE generating spherical HDL particles, that follow subsequent interchange of lipids with other lipoproteins (VLDL, LDL) mediated by cholesterol ester transfer protein (CETP) and phospholipid transfer protein (PLTP).<sup>26,29</sup>

While the causal involvement of LDL in coronary disease and the associated risk of myocardial infarction has been strongly supported by genetic studies,<sup>30,31</sup> the association between HDL cholesterol levels and the risk of myocardial infarction is substantially more complex.<sup>30</sup> Although epidemiological studies consistently showed an inverse relation between HDL cholesterol (HDL<sub>chol</sub>) plasma levels and the risk of coronary disease or myocardial infarction in the primary prevention setting,<sup>32,33</sup> the association between HDL<sub>chol</sub> plasma levels and cardiovascular events is likely altered in patients with established coronary disease.<sup>34,35</sup>

Moreover, genetic Mendelian randomization analyses examining the relation of polymorphisms (SNPs) linked to changes in HDL<sub>chol</sub> did not find a consistent association with an altered risk of myocardial infarction, whereas LDL<sub>chol</sub> SNPs were consistently associated with the risk of myocardial infarction.<sup>30</sup> Furthermore, genome wide association scan (GWAS) studies showed an increased frequency of 11 LDL<sub>chol</sub>-associated variants in a sample of patients with coronary artery disease (CAD) vs. controls that was not reported for HDL<sub>chol</sub>-associated variants.<sup>36</sup> In addition, heterozygotes carriers of ABCA1 mutations with a moderately decreased of HDL<sub>chol</sub> did not have an association with an increased risk of ischaemic heart disease.<sup>37</sup>

Moreover, recent clinical trials evaluating HDL<sub>chol</sub>-raising therapies such as the CETP inhibitors torcetrapib and dalcetrapib or niacin (with laropiprant) did not demonstrate a reduced risk of cardiovascular events in patients with coronary disease.<sup>38–42</sup> In fact, both clinical and genetic studies suggest that HDL<sub>chol</sub> alone is not a sufficient therapeutic target and that alterations of HDL function in patients with coronary disease and in chronic inflammatory conditions likely need to be considered. In this respect, the lack of improved endothelial function after dalcetrapib treatment suggests that raising HDL<sub>chol</sub> levels alone cannot restore endothelial function.<sup>43</sup>

An important factor to consider while evaluating these studies is that unlike the genetic randomized studies, most of the clinical trials have involved a markedly higher number of men both in the healthy and disease groups (~80% men). As we will discuss in more detail, there are likely significant gender differences with respect to HDL levels, composition, and metabolism that may be of relevance in this context.

Of note, the continuous remodelling and interchange of components generates a very heterogeneous population of HDL particles in terms of size, composition, and functionality.<sup>26,44</sup> Various factors contribute to the heterogeneity of HDL particles and the existence of multiple subsets of particles (Figure 1):

- (i) combinations of several exchangeable apolipoproteins (apoA-I, apoA-II, and apoE as major constituents) and their capacity to dissociate and/or interchange between particles;<sup>45,46</sup>
- (ii) high plasticity of apolipoproteins with constant conformational changes, induced by HDL particle composition and size, that could affect interactions with other proteins and therefore their functionality;<sup>47–50</sup>
- (iii) numerous additional proteins associated to specific HDL particles that accomplish multiple functions;<sup>51,52</sup>
- (iv) a complex set of lipid species that not only act as an inert cargo but also can activate different signalling pathways in numerous cell types.<sup>53</sup>

## 3. Effects of HDL from healthy subjects or reconstituted HDL on endothelial cell function

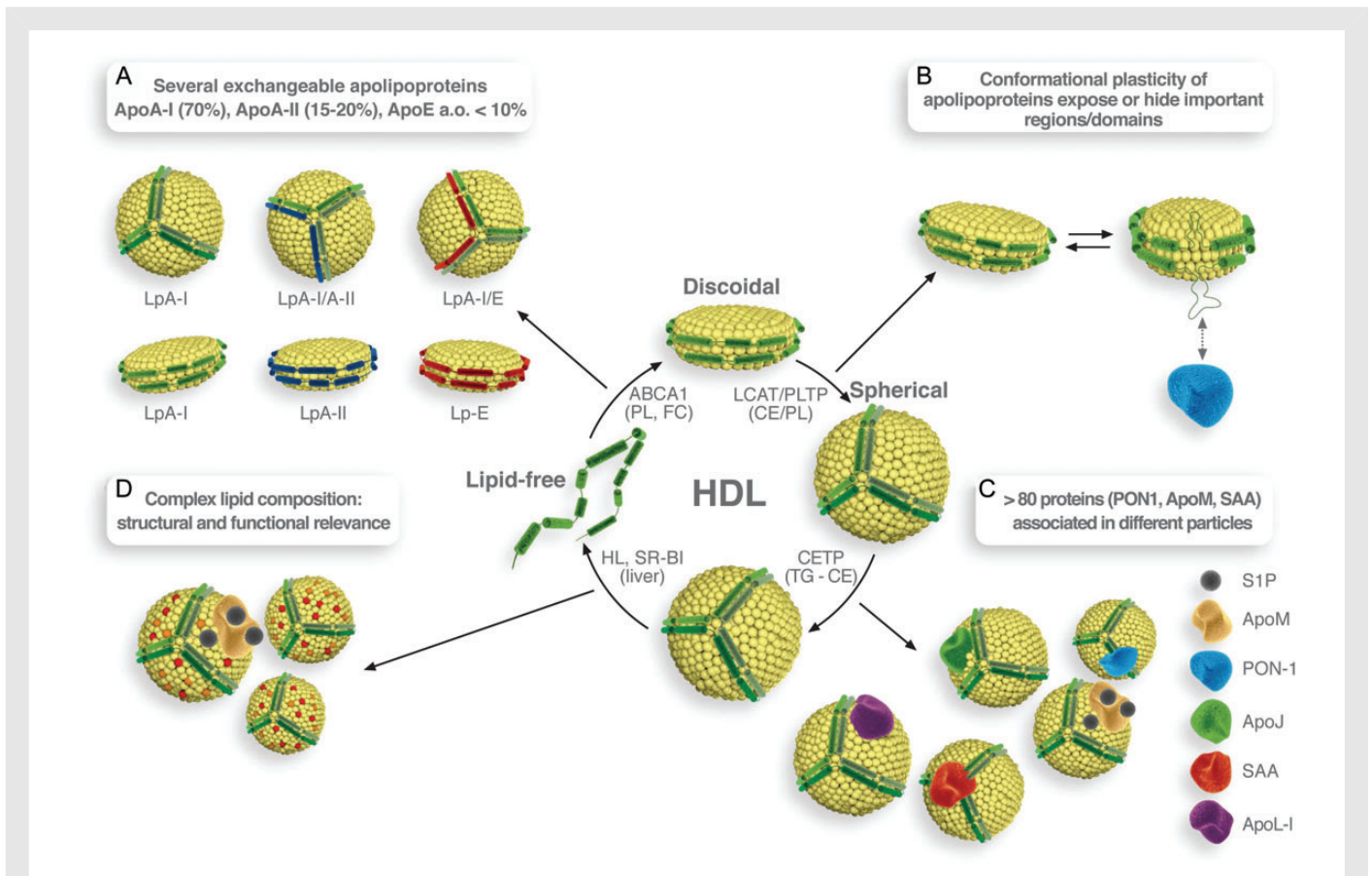
Endothelial cells (EC) are highly exposed to HDL, both at the luminal side, i.e. in contact with the circulating lipoprotein, as well as from the subendothelial side.<sup>54,55</sup> ApoA-I and HDL can be transferred through the endothelium via transcytosis, being internalized on the luminal side and released into the arterial intima.<sup>56</sup> Two different pathways have been described, lipid-poor ApoA-I internalization and transcytosis are ABCA1-mediated, whereas HDL particle internalization is dependent on SR-BI and ABCG1.<sup>54,55,57</sup> Additionally, endothelial lipase (EL) has been also implicated in the HDL transcytosis process.<sup>58</sup> Lymphatic vessels express SR-BI and have recently been shown to play a role in the transport of HDL back to the circulation.<sup>59,60</sup>

### 3.1 Effects of HDL on endothelial NO production

#### 3.1.1 Mechanisms whereby HDL may stimulate endothelial NO production

Production of the vasodilator NO by endothelial nitric oxide synthase (eNOS) is a highly regulated process and important for vascular homeostasis.<sup>61</sup> HDL modulates eNOS activity as has been demonstrated in *in vitro* studies using human primary endothelial cells,<sup>23</sup> but also in *in vivo* studies using animal models<sup>23,62</sup> and in humans after applying intravenous rHDL infusion.<sup>63</sup> HDL has been shown to stimulate endothelial cell eNOS activity through the endothelial SR-BI receptor.<sup>64</sup> In recent years, more detailed molecular mechanisms of SR-BI-mediated endothelial signalling pathways have been delineated involving sequential activation of Src Tyrosine kinase, PI-3K, Akt kinase, and Erk1/2 MAPK leading to activation of eNOS by phosphorylation of the enzyme at Ser-1177<sup>65</sup> (Figure 2).

SR-BI interaction with the N-terminal PDZ domain of PDZK1 is required in order to mediate activation of eNOS by HDL.<sup>66</sup> Studies using SR-BI and its homologue CD36 fusion proteins identified the SR-BI domain as responsible for HDL-mediated eNOS activation located at the second transmembrane (TM) domain and its C-terminus.<sup>67</sup> Although deletion of this domain did not impair cholesterol efflux to HDL, the efflux seems to be required since cholesterol-free cyclodextrin was replicating the eNOS activation effect while



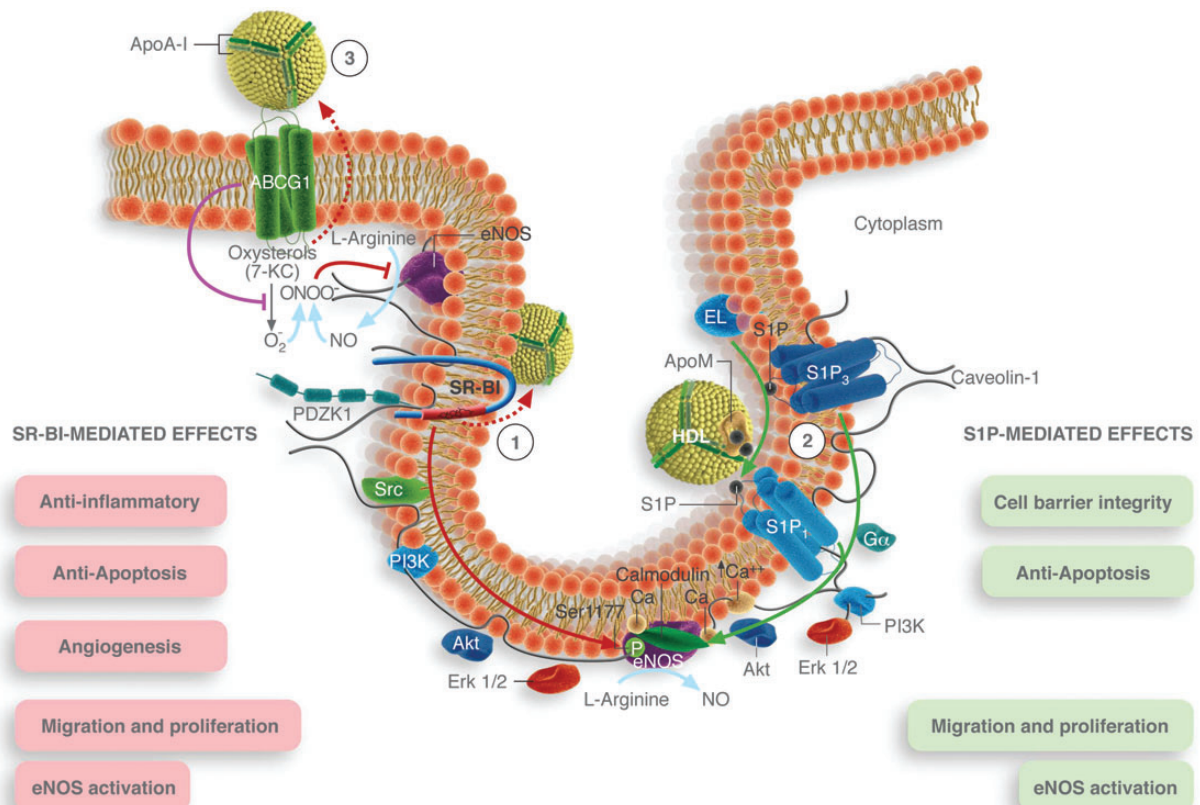
**Figure 1** HDL particle complexity and heterogeneity. Normal HDL metabolism (central panel) involves modulation of the composition and structure of the lipoprotein: apolipoprotein secretion mainly by the liver and intestine; assembly of discoidal HDL by PL and FC lipidation; further lipid enrichment in CE and PL by LCAT and PLTP action, respectively, raising small spherical HDL; exchange of CE with TG with VLDL/LDL mediated by CETP increasing the size of HDL particles; and delivery of CE, TG and PL to the liver by action of HL and SR-BI. Additional properties increase the complexity of HDL by generation of a large set of particles: (A) The availability of several exchangeable apolipoproteins (ApoA-I, ApoA-II, ApoE, and to a lesser extension ApoA-IV) present in HDL particles as different combinations may entail different functionalities. (B) Each of these exchangeable apolipoproteins is highly dynamic leading to changes of its tertiary structure upon changes of HDL particle composition. These structural modifications can expose or hide different regions/domains of the protein that could be potentially important for interaction with other proteins. (C) More than 80 proteins have been identified to be associated with HDL, including: ApoM (~5% of all HDL particles), PON-1 (10–12% of all HDL particles), ApoL-I, SAA, or Clusterin (ApoJ). Each of these proteins accomplishes specific functions that have been associated with HDL functions, for example, the antioxidative properties of PON-1 or the immune functions of ApoL-I in fighting *Trypanosoma* infection. (D) Further heterogeneity derives from different lipid compositions of HDL particles. Lipids carried by HDL are not only a mere cargo but can play important roles for HDL function. A well-characterized example is sphingolipid S1P, a crucial player for HDL-mediated signal transduction to numerous cell types. Interestingly, ApoM is required for HDL being a carrier of S1P, and this protein is only present in about 5% of HDL particles. LpA-I: lipoprotein A-I; PL: phospholipids; FC: free cholesterol; LCAT: lecithin cholesterol acetyltransferase; CE: cholesterol ester; PLTP: phospholipid transfer protein; TG: triglyceride; HL: hormone sensitive lipase; PON-1: Paraoxonase 1; ApoJ: Clusterin; SAA: serum amyloid A; S1P: sphingosine 1 phosphate.

cholesterol-loaded cyclodextrin did not.<sup>67</sup> Finally, recent studies defined the function of the second TM domain of SR-BI as a cholesterol sensor. A single mutation (Q445A) on this TM domain reduced its binding to cholesterol by 71% and this resulted in an impaired HDL-induced signalling without altering cholesterol efflux to HDL.<sup>68</sup> This transmembrane domain lacks homology with known cholesterol-binding domains such as 3-hydroxy-3-methylglutaryl-CoA reductase, liver X receptors, sterol regulatory element-binding protein, or Niemann-Pick C1 and C2,<sup>69</sup> being the first sterol-sensing protein to regulate kinase signalling.<sup>68</sup> The mechanism transferring the signal from SR-BI cholesterol sensor to the Src kinase is still not defined.

Another important mechanism regulating eNOS activation involves HDL-associated sphingosine 1 Phosphate (S1P).<sup>70–72</sup> Both S1P<sub>1</sub> and

S1P<sub>3</sub> receptors have been related to HDL-mediated NO release via intracellular Ca<sup>2+</sup> mobilization and PI-3K/Akt pathway activation<sup>72,73</sup> (Figure 2). Ca<sup>2+</sup>/calmodulin activation seems to disrupt the inhibitory interaction of eNOS with caveolin promoting NO production.<sup>71,74–76</sup> Activation of G-proteins by the receptors induces PI3K and Akt pathways resulting in phosphorylation of eNOS Ser 1177 and activation.<sup>77,78</sup> HDL is the main carrier of plasma S1P and apolipoprotein M (ApoM) is considered as the S1P binding protein on HDL. Liver-specific ApoM transgenic animals showed S1P associated to a subset of large HDL particles and lack of ApoM on ApoM KO leads to dysfunctional endothelial barrier function in the lung, presumably related to a loss of S1P on HDL although other cellular specific mechanisms cannot be excluded.<sup>79–83</sup> These studies suggest a preferential role of ApoM on the S1P





**Figure 2** Signal transduction pathways regulating HDL homeostatic physiology in endothelial cells. (1) HDL-mediated signalling by SR-BI requires efflux of cholesterol towards the ApoA-I-bound HDL, which in turn modulates cholesterol sensor activity located at SR-BI 2nd TM domain. The signal is transduced to activate Src Tyr kinase and a downstream cascade involving PI3K and MAPK signalling that results in phosphorylation of eNOS Ser1177 inducing its activation. SR-BI C-terminal interaction with PDZK1 is required for signal transduction. Besides eNOS activation, this pathway initiates other potential anti-atherogenic endothelial functions (see panels). (2) HDL/ApoM-bound S1P signals through S1P1/P3 receptors, located within the caveolae, while EL may be mediating S1P release from HDL facilitating the binding to the receptor(s). S1P1/P3 initiates signalling through G-protein coupled receptors, followed by downstream activation of PI3K, AKT, and MAPK pathways and activating phosphorylation of eNOS. S1P receptors also induce an increase of intracellular  $\text{Ca}^{++}$  levels with calmodulin activation that is able to displace caveolin-1 from its inhibitory complex with eNOS. (3) HDL induces endothelial ABCG1-mediated efflux of oxysterols (like 7-ketocholesterol, 7-KC) and can thereby prevent inhibition of eNOS. SR-BI, scavenger receptor BI; Src: PI3K: phosphoinositide-3-kinase; ERK, (mitogen-activated protein kinase) extracellular-signal-regulated kinases; eNOS, endothelial nitric oxide synthase; EL, endothelial lipase; S1P, sphingosine 1 phosphate; ApoM, apolipoprotein M; Ca, calcium; S1P1 and S1P3, sphingosine 1 and sphingosine 3 receptor; ABCG1, ATP-binding cassette transporter G1; 7-KC, 7-ketocholesterol; NO, nitric oxide; PDZK1, PDZ domain containing 1.

metabolism but further studies will have to address their relevance in human physiology.

### 3.1.2 Mechanisms whereby HDL may prevent reduction of endothelial NO availability

Oxidized LDL (oxLDL) treatment induces displacement of eNOS from its functional subcellular location, caveolae, and inhibits NO production.<sup>84</sup> HDL has been shown to counteract the oxLDL effect by preserving eNOS location and activity.<sup>85</sup> More recently, it was shown that HDL prevents endothelial dysfunction via endothelial ABCG1-mediated efflux of cholesterol and 7-oxysterols,<sup>86</sup> in part by decreasing the interaction of caveolin-1 and endothelial NO synthase.<sup>87</sup>

In addition, HDL has also been shown to modulate NO production by increasing eNOS stability via a mechanism involving MAPK and PI3K/Akt pathways.<sup>88</sup>

## 3.2 HDL: role for maintenance of endothelial integrity

HDL may protect endothelial integrity by promotion of endothelial repair responses after vascular injury and by reducing endothelial cell apoptosis.<sup>23,24</sup> Endothelial cell apoptosis can be induced by oxLDL, TNF $\alpha$ , and other factors, which may also lead to disruption of endothelial monolayer integrity.<sup>89–91</sup> HDL has been proposed to protect vascular integrity by different mechanisms.<sup>90,92,93</sup>

S1P bound to HDL has been implicated in endothelial barrier homeostasis by increasing lipid raft number as well as Akt-mediated eNOS activation in an enhanced way in comparison to albumin-bound S1P.<sup>94</sup> Small HDL3 particles are enriched on S1P, have increased S1P/SM molar ratio and potentially attenuated apoptosis in endothelial cells, and delayed LDL oxidation, the S1P/SM molar ratio was positively correlated with the anti-apoptotic and antioxidative activities of HDL.<sup>95</sup>

S1P also plays a role in migration and proliferation and accordingly in wound healing through S1P1 and S1P3 receptors and downstream activation of the ERK pathway, possibly ligated to eNOS activation.<sup>96,97</sup> Moreover, we have recently observed that HDL-associated clusterin may contribute to endothelial anti-apoptotic effects of HDL.<sup>24</sup>

HDL also has been shown to stimulate endothelial cell migration and an anti-apoptotic effect through binding to SR-BI that was at least partially independent of eNOS as demonstrated by eNOS knockdown studies.<sup>24,98</sup>

The anti-apoptotic effect of HDL has been observed in all of its different subfractions upon treatment of microvascular endothelial cells with oxLDL, but the lipid-poor apoA-I fraction showed a more-pronounced effect.<sup>99</sup>

### 3.3 HDL and endothelial anti-inflammatory effects

HDL has been shown to reduce endothelial cell adhesion molecule expression and monocyte adhesion. Down-regulation of VCAM-1, ICAM-1, and E-selectin by HDL is mediated by the SR-BI and S1P receptors transduction signal pathways that has been described earlier.<sup>100–102</sup> The anti-inflammatory capacity of HDL has been assigned to its phospholipid content in addition to apoA-I.<sup>103</sup>

Another mechanism in small, lipid-poor HDL (HDL3 or nascent HDL) and apoA-I seems to be mediated in most cells, including endothelial cells, immune cells (macrophages, monocytes, and T cells etc.) via cholesterol depletion of lipid rafts.<sup>29</sup>

Moreover, the ATP-binding cassette (ABC) cholesterol efflux transporters, such as ABCA1 and ABCG-1, have been suggested to exert anti-inflammatory functions.<sup>104,105</sup> Furthermore, down-regulation of the infiltration of neutrophils/monocytes into the media/intima of the arterial wall by HDL has been related to decreasing of the surface expression of integrin CD11b.<sup>101,106</sup> This has been partly implied to be mediated by apoE, which is also responsible for diminishing the lipid load within monocytes.<sup>107</sup>

Additionally, it has been shown that lipid-free apoA-I decreases vascular endothelial inflammatory activation through up-regulation of 24-dehydrocholesterol reductase (DHCR24)<sup>108</sup> and the well-known anti-inflammatory protein heme oxygenase I (HO-1), which is mediated through PI3K/AKT pathway being one of the pathways that are activated via HDL in an SR-B1-dependent manner. This mechanism has been observed to act independent of the down-regulation of TNF $\alpha$ -mediated increase of ICAM-1 and VCAM-1 by inhibiting the NF- $\kappa$ B pathway.<sup>109</sup>

HDL can protect LDL from oxidation and thereby reduce its impact on endothelial cell inflammatory activation. The lipoprotein oxidation is in part mediated via oxidants such as hypochlorous acid (HOCl) produced by myeloperoxidase (MPO). The antioxidant effect of HDL is partially mediated by the paraoxonase and arylesterase activity of HDL-associated enzyme paraoxonase-1 (PON1).<sup>110</sup> Oxidation of LDL has been inhibited by PON1<sup>111</sup> and the same has been observed with respect to oxidation of HDL.<sup>110</sup> Most profound antioxidant capacities have been associated with the dense HDL subpopulations (HDL3) with a high capacity to attenuate LDL oxidation. Proteomics analysis identified, in this subpopulation, other putative antioxidant proteins associated to HDL as platelet-activated factor acetyl-hydrolase (PAF-AH), apoL-1 (a neutralizer of *Trypanosoma brucei*)<sup>112</sup>, apoF, PLTP, apoJ/Clusterin, and PON3.<sup>51</sup> Other proteomic studies underline the role of HDL in regulation of the complement system as well as inhibiting protease action and thus underlining its anti-inflammatory role.<sup>52</sup> Recently, our group has also

shown that PON-1 is an important determinant for the capacity of HDL to stimulate endothelial NO production.<sup>23</sup> HDL added to a co-culture of LDL and monocytes was able to prevent the oxidation of the LDL mediated through the inhibition of MCP-1 on human aortic endothelial cells (HAEC).<sup>113</sup>

### 3.4 HDL regulation of endothelial thrombotic activation

An anti-thrombotic effect of HDL in humans was suggested by a study in healthy subjects where infusion of reconstituted HDL limited their pro-coagulant state after endotoxin exposure.<sup>114</sup> Furthermore, a potential anti-thrombotic capacity of HDL was observed in an acute arterial thrombosis rat model after infusion of apoA-I Milano, which showed an extended time of thrombus formation.<sup>115</sup> Several molecular mechanisms have been proposed involving processes independent or dependent of NO production.<sup>116</sup> Prostacyclin (PGI<sub>2</sub>) is a vasoactive prostaglandin that acts synergistically with NO in vasomotor control, inhibition of platelet activation, and attenuation of smooth muscle cell proliferation. Prostacyclin expression is induced by native HDL in endothelial cells,<sup>117,118</sup> and to a lesser extent using delipidated HDL.<sup>119</sup> HDL-induced expression of prostacyclin is mediated through provision of arachidonate<sup>117,120</sup> or in part by induced Cox-2 expression.<sup>121,122</sup> As discussed earlier, HDL transports several types of sphingolipids<sup>121</sup> such as glucosylceramide that was reported to be low in plasma of venous thrombosis patients.<sup>123</sup> LDL and VLDL have more than five-fold higher content of ceramide than HDL.<sup>124</sup> Sphingosine inhibits prothrombin activation<sup>125</sup> and most of its effects are mediated via its G-protein coupled receptors.<sup>126</sup> As it has been shown that apoptosis of endothelial cells promotes thrombosis,<sup>127</sup> one may speculate that there is an implication that the anti-apoptotic effects of HDL described earlier may contribute to reduced thrombosis.<sup>128</sup>

## 4. Gender differences of HDL

In the pre-menopausal state, women have lower LDL-C levels and higher HDL-C levels when compared with men,<sup>129</sup> which has been reported to change after menopause.<sup>130</sup> This has been mainly related to sex hormones, such as estrogens, that behave as LDL-R up-regulating agents.<sup>131</sup> In addition to the expected sex differences in concentrations of triglycerides, LDL-cholesterol, and HDL-cholesterol, women also had a different subclass profile consisting of larger LDL and HDL particles. The gender difference was most pronounced for HDL, with women having a two-fold higher (8 vs. 4  $\mu$ mol/L) concentration of large HDL particles than men.<sup>132</sup>

HDL-associated estradiol has been suggested to stimulate endothelial cell eNOS and thereby endothelium-dependent vasodilation. This could be seen in pre-menopausal women, but also in post-menopausal women receiving hormone replacement therapy,<sup>133</sup> supporting the possibility that sex hormones may impact on vascular effects of HDL, including direct association with the particle. Most studies have not examined gender differences of HDL function and composition, so that this important topic has to be studied in more detail in the future.

Another interesting aspect with respect to gender differences and their potential impact on HDL-mediated effects on endothelial cells is the identification of the X-linked inhibitor of apoptosis proteins (XIAP).<sup>134</sup> Such genes could potentially play a role in different vascular effects of lipoproteins between men and women.

## 5. Alterations of endothelial effects of HDL from patients with coronary artery disease

Accumulating evidence has indicated that composition and vascular effects of HDL are markedly altered as part of the acute-phase immune response. HDL during the acute-phase response to inflammation gets enriched in acute-phase proteins, such as SAA, and becomes pro-inflammatory, i.e. stimulates monocyte chemotactic protein 1 (MCP1) expression.<sup>135</sup> HDL has therefore by some investigators been considered of having a function to modulate the inflammatory acute-phase response to inflammation *in vivo*.

Importantly, HDL isolated from patients with coronary disease (in contrast to HDL from healthy subjects) exhibited a pro-inflammatory rather than an anti-inflammatory phenotype when exposed to endothelial cells.<sup>22</sup> Moreover, HDL from patients with stable CAD (sCAD) or an acute coronary syndrome (ACS), in contrast to HDL from healthy subjects, failed to stimulate endothelial cell NO production, and subsequently loss of the capacity to promote endothelial repair, further demonstrating that the quality of HDL with respect to its endothelial effects is markedly altered in patients with sCAD (Table 1).<sup>23</sup>

In these studies, healthy subjects were defined as individuals without established cardiovascular disease and without cardiovascular risk factors. Stable CAD (sCAD) was defined as patients with at least one >50% coronary stenosis and patients with acute coronary syndrome (ACS) that were hospitalized for an acute myocardial infarction. The above observations raised the question of underlying mechanisms leading to these profound alterations of endothelial effects of HDL in patients with coronary disease. Of note, HDL-associated paraoxonase-1 activity is markedly reduced in patients with sCAD, likely allowing a more rapid lipid oxidation of the lipoprotein as indicated by increased MDA-content of HDL in these patients.<sup>23</sup> Notably, HDL from patients with sCAD had an increased binding affinity to the lectin-like oxidized LDL receptor 1 (LOX-1) which signals via PKC $\beta$  pathway to reduce eNOS activity<sup>23</sup> by promoting the inhibitory eNOS phosphorylation at thr-495 (Figure 3).

Interestingly, HDL serves as a scaffold upon which MPO and PON1 interact during inflammation, generating a ternary complex during acute inflammation that shows reciprocal regulation of enzymatic

activities where PON1 partially inhibits MPO oxidative activity while MPO can inactivate PON1.<sup>136</sup> MPO oxidizes Tyr71 of PON1, a critical residue for its HDL binding and activity displacing the balance to a more oxidative state (Figure 3).

Myeloperoxidase (MPO) is a heme peroxidase expressed by neutrophils, monocytes, and activated macrophages in atherosclerotic plaques.<sup>137–139</sup> MPO enzymatic production of various reactive oxidants and radical species contributes to the elimination of invading parasites and pathogens.<sup>140</sup> However, in a number of chronic inflammatory diseases, including atherosclerosis, the extended oxidative activity of MPO promotes lipid peroxidation and protein modifications leading to host tissue injury.<sup>141,142</sup> MPO enzymatic activity is very complex with multitude of substrates that generates multiple reactive species and a variety of post-translational modifications: oxidation, chlorination (hypochlorous acid –HOCl– via MPO/H<sub>2</sub>O<sub>2</sub>/Cl<sup>–</sup> system), nitration (peroxynitrite –ONOO– via MPO/H<sub>2</sub>O<sub>2</sub>/NO), and carbamylation (via MPO/H<sub>2</sub>O<sub>2</sub>/thiocyanate –SCN<sup>–</sup>).<sup>143,144</sup>

Direct interactions between MPO and apoA-I have been reported suggesting that apoA-I is a main target of MPO-derived reactive species due to their short lifespan and high reactivity with the first available residue.<sup>145</sup> Indeed, analysis of human arterial atheroma plaques has identified numerous modifications in apoA-I associated with impairment on potential athero-protective properties of the HDL particle.<sup>145–147</sup> *In vitro* chlorination or nitration of HDL and lipid-free apoA-I inhibited ABCA1-dependent cholesterol efflux from macrophages.<sup>145</sup> In a similar approach, chlorination of HDL from healthy donors induced a reduced wound healing capacity of endothelial cells.<sup>148</sup> HDL carbamylation impaired activation of LCAT, PON1 functionality, and antioxidant HDL capacity.<sup>149</sup> A specific antibody identified oxTrp72 residue of apoA-I as an abundant post-translational modification (20% of total apoA-I) in human atheroma, but surprisingly, virtually all the oxTrp72-apoA-I molecules recovered were in a lipid-poor form and not associated with HDL particles.<sup>150</sup> Moreover, apoA-I harbouring oxTrp72 modification showed no detectable ABCA1-dependent cholesterol efflux activity, impaired HDL biogenesis capacity, and a pro-inflammatory effect on endothelial cells inducing expression of VCAM-1 and increased activation of NF- $\kappa$ B.<sup>150</sup>

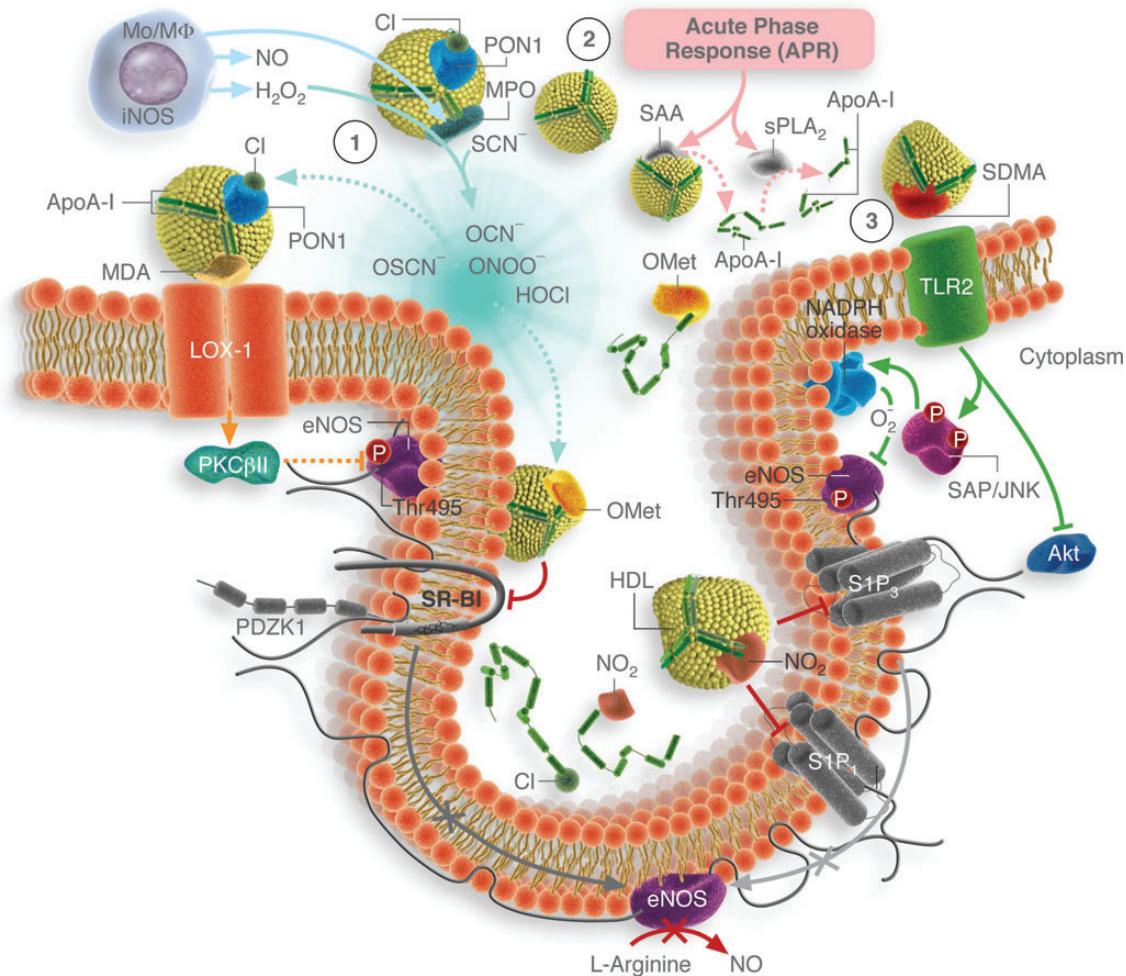
Moreover, in recent proteomics studies we and others have observed marked alterations of the HDL proteome in patients with CAD.<sup>24,52</sup>

**Table 1 HDL function (with a focus on endothelial effects) in patients with CAD, CKD, and healthy subjects**

Effect of HDL on	Healthy	CAD	CKD	Proposed mechanism of HDL dysfunction
Endothelial cell NO production	Induced	Not changed	Inhibited	Oxidation of ApoA-I protein LOX-1-mediated TLR2/NADPH oxidase-mediated
Endothelial cell inflammatory activation	Anti-inflammatory	Loss of anti-inflammatory effects	Pro-inflammatory	SAA displacement of ApoA-I, SDMA accumulation in HDL
Endothelial cell antioxidant capacity	Induced/Functional	Impaired	Impaired	Reduced PON1 activity (MPO oxidative modification)
Endothelial cell apoptosis	Anti-apoptotic	Pro-apoptotic	ND	
Capacity to promote endothelial cell migration and endothelial repair	Induced	Impaired	Impaired	Oxidation of ApoA-I protein
Regulation of endothelial cell thrombotic activation	Anti-thrombotic	Potentially Pro-thrombotic	ND	Lipid-oxidized species (lipid peroxides)

ND, not determined.





**Figure 3** Mechanisms of altered endothelial effects of HDL from patients with coronary disease or increased cardiovascular risk. (1) Monocytes/Macrophages (Mφ) can release MPO, an oxidative enzyme leading to modification of HDLs lipid and protein components such as PON-1 or apoA-I altering their function. MPO uses substrates such as NO or H<sub>2</sub>O<sub>2</sub> produced by activated monocytes/macrophages or exogenous chemicals (as SCN<sup>-</sup> derived from cigarette smoke) to generate reactive species. Nitrosylation, chlorination, or methylation are some of the modifications of apoA-I leading to dysfunctional SR-BI and S1P receptors signal transduction mediated by HDL. Malondialdehyde (MDA) is one of the oxidized products that accumulate on HDL when PON-1 antioxidant enzyme is not functional. MDA-bound HDL can activate endothelial LOX-1 and induce PKCβII activation, leading to inhibitory phosphorylation at Thr495 of eNOS. (2) During acute-phase SAA protein plasma levels increase drastically and can displace ApoA-I from HDL particles. Although SAA accomplishes an important role in innate immune defense, chronic inflammation leads to a dysfunctional HDL unable to perform homeostatic functions. (3) HDL-bound SDMA activates TLR2 (independent of TLR1 or TLR6) and induces ROS production via NADPH oxidase, which impairs eNOS activation in parallel to AKT inactivation. MPO-Mieloperoxidase; PON-1-Paroxonase-1; ApoA-I, Apolipoprotein AI; SAA, serum amyloid A; sPLA<sub>2</sub>, secretory phospholipase A<sub>2</sub>; TLR2, toll-like receptor 2; SDMA, symmetric dimethylarginine; eNOS, endothelial nitric oxide synthase; RCT, reverse cholesterol transport.

In particular, HDL in patients with CAD had higher amounts of apoCIII, whereas the HDL-bound clusterin amounts were reduced. These alterations in the HDL proteome were found to impact on the effects of HDL on endothelial cell apoptosis, i.e. HDL from healthy subjects exerted endothelial cell anti-apoptotic effects *in vitro* and *in vivo*, whereas HDL from patients with CAD had markedly attenuated anti-apoptotic properties.<sup>24</sup>

Further studies have suggested that in particular a specific subset of STEMI patients with significant acute-phase inflammatory response showed a most defective HDL stimulation of endothelial NO production.<sup>151</sup>

Additionally, HDL from patients with chronic kidney disease, that have a markedly elevated cardiovascular risk, promoted (in contrast to

HDL from healthy subjects) endothelial superoxide production leading to reduction of endothelial nitric oxide (NO) bioavailability, an effect mediated via toll-like receptor-2 (TLR-2), providing a link of altered HDL function to activation of pathways of innate immunity on endothelial cells.<sup>152</sup>

Activation of TLR-2 by HDL from patients with CKD was at least in part mediated by HDL-bound SDMA, i.e. a small molecule (Figure 3). HDL from patients with CKD have also shown reduced antioxidant and anti-inflammatory activities and impaired endothelial repair capacities compared with HDL from healthy subjects.<sup>152,153</sup>

In contrast, HDL from healthy subjects has been observed to limit TLR-dependent inflammatory activation of macrophages and the molecular mechanism involves induction of the transcriptional regulator



ATF3 by HDL that down-regulates the expression of toll-like receptor (TLR)-induced pro-inflammatory cytokines.<sup>154</sup>

In patients with type-2 diabetes, that are also at increased risk of cardiovascular events, the capacity of HDL to reverse the inhibitory effect of oxLDL on endothelium-dependent arterial relaxation was impaired.<sup>155,156</sup> In these patients, HDL also failed to stimulate endothelial NO production.<sup>157</sup>

## 6. Summary and conclusions

HDL can exert numerous effects on endothelial cell functions, including modulation of endothelial cell nitric oxide availability, endothelial repair and endothelial cell apoptosis, endothelial adhesion molecule/chemokine expression, and endothelial pro-thrombotic activation. The underlying molecular mechanisms are now better understood, and involve SR-BI receptor and S1P3-receptor-dependent endothelial signalling as well as ABCG-1 dependent efflux of oxysterols. Importantly, however, several studies over the past years have clearly demonstrated that the endothelial effects of HDL are highly heterogeneous and are altered in particular in patients with coronary disease or an increased cardiovascular risk. The underlying mechanisms remain to be fully explored, but involve post-translational modifications of apoA-I and paraoxonase-1, important modulations of the HDL proteome and liposome. Whereas myeloperoxidase-dependent modification of HDL-associated proteins likely plays an important role of post-translational alterations, the acute-phase response clearly modulates the HDL proteome that may contribute to altered properties of HDL in inflammatory diseases.

This loss of HDL functionality to protect the endothelium may facilitate common cardiovascular risk factors (smoking, hypertension, hypercholesterolaemia, type-2 diabetes, and ageing) to promote altered arterial endothelial cell functions.

In this review, we underlined the differences of effects of HDL on the endothelium in different clinical settings and their dependence on the vast heterogeneity of the HDL particles under (patho)physiological states. In fact, proteomics and lipidomics have brought novel insights into the complexity of HDL changing our concept/perspective from a simple model of a homogeneous HDL cholesterol carrier to a dynamic array of particles with different components and functionalities. However, many challenges have to be overcome including the development of standardized methodology for HDL isolation and a systematic nomenclature in order to facilitate comparisons between different isolation methods and studies. Another challenge in the assessment of HDL composition and functions is to identify and address potential artefacts related to HDL isolation methods.<sup>48,158,159</sup> One potential step to reduce HDL changes during isolation is the use of heavy water (D<sub>2</sub>O) in the ultracentrifugation fractionation that can minimize oxidation artefacts.<sup>160,161</sup>

Moreover, new insights on the structural basis of ApoA-I and its highly dynamic conformations can provide a better understanding of HDL functions, however, the vast HDL particle heterogeneity imposes limitations to higher resolution protein structures. Advances of lipoprotein structure resolution combined with analysis of specific oxidized modifications will help to understand which proteins and functions are impaired under the pro-oxidative conditions of the atherosclerotic plaque.

HDL exposure to chronic or acute inflammation leads to profound changes of HDL, turning HDL towards a pro-inflammatory particle. It has been proposed that this acute-phase HDL plays a role in the innate

immune response to fight against pathogen infections. However, during atherosclerosis or coronary disease, this 'activated' HDL phenotype may be partially chronically maintained as a result of unresolved inflammation impairing its homeostatic atheroprotective functions. A better understanding on the resolution of acute response and the switch between these different HDL phenotypes could provide a better understanding of HDL changes in chronic inflammatory diseases.

Recent pharmacological and genetic studies suggest that modulation of HDL cholesterol levels alone is not a sufficient therapeutic target to provide protection from cardiovascular events. However, understanding the mechanisms leading to altered vascular effects of HDL may lead to potential novel therapeutic measures for the prevention of progression of coronary disease.

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